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14. ABSTRACT
We tested whether metabolic inhibitors, metformin or 2-deoxy-glucose, function synergistically with docetaxel to block prostate cancer cell proliferation. Cell proliferation curves allowed calculation of the combination index, a quantitative measure of drug interactions, for LNCaP or PC-3 cell lines. Data trended toward synergy between microtubule-targeted drugs and metabolic inhibitors. Synergy was consistently calculated at the ED50 for each drug, but the combination index often shifted toward antagonism at higher drug doses. Statistical analyses indicated that some drug combination data were not significantly different from either drug alone, reducing confidence in the overall conclusion of synergy.

15. SUBJECT TERMS
docetaxel, metformin, 2-deoxy-glucose, LNCaP, PC-3, cell proliferation, combination index

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Introduction

Prostate cancer is the second leading cause of cancer deaths for men in the United States (American Cancer Society, 2013 statistics). Although often cured by surgery or radiation treatment, for many patients the disease progresses from one responsive to androgen removal to one where cancer cells have become androgen insensitive. This latter stage is referred to as metastatic castrate-resistant prostate cancer (mCRPC). The current chemotherapy treatment for mCRPC is docetaxel, but this drug treatment is not very effective, increasing lifespan by an average of 3 months (reviewed by 1,2). Docetaxel (Taxotere™) and other taxanes are drugs that act by stabilizing microtubules of the mitotic spindle, blocking cell division and ultimately leading to cell death (3). An alternative to blocking cells in mitosis is to inhibit intracellular metabolism. Many cancers depend on glycolysis for most ATP production (the Warburg effect) and these high glycolytic rates also fuel the pentose phosphate pathway for biosynthesis of nucleosides and amino acids, suggesting that metabolic inhibitors may induce cell death in cancer cells (4,5), and this has been demonstrated in several prostate cancer cell lines treated with 2-deoxyglucose (2-DG), an inhibitor of glycolysis (6). Metformin is another metabolic regulator currently under study as an anticancer drug because of the strong epidemiological data showing that diabetics taking metformin have a significantly reduced risk of cancer development, compared to diabetics taking other types of medication (7). Use of metformin to treat cancers is currently under investigation, and has shown the ability to induce cell death in cell lines from prostate (6), breast (8) and pancreatic (9) cancers. The experiments performed here were designed to test whether the combination of microtubule-targeted drugs and metabolic inhibitors act synergistically (more-than-additive) to inhibit proliferation of human prostate cancer cells grown in cell culture. Experiments were performed in two commonly used human prostate cancer cell lines, LNCaP and PC-3. LNCaP cells are androgen-sensitive human prostate adenocarcinoma epithelial cells that have a low metastatic potential. PC-3 cells are androgen-insensitive human cells and have a high metastatic potential; these cells are a model for mCRPC (10).

Body

The original two goals summarized in the SOW were to address the following questions:

A. Does increased microtubule stability decrease glycolytic rate or the pentose phosphate pathway?
B. Does microtubule stabilization with docetaxel synergize with metformin or 2-deoxy-glucose to inhibit cell proliferation and/or induce cell death?

We opted to begin with Goal B because completion of this goal would tell us whether testing a possible mechanism responsible for the synergy (Goal A) was necessary. Given the 1 year time frame of the Exploration-Hypothesis award, we were able to thoroughly explore Goal B, but did not have the time or resources to explore Goal A. We also realized that we would need to scale back to 2 cell lines in order to conduct experiments that thoroughly explored each drug concentration series and that allowed sufficient time to replicate all experiments several times for confidence in the reproducibility and statistical significance of the work.

Our results are summarized below in three major sections: Methods, Results and Interpretations, and Conclusions. Figures and Tables are included at the end of the report.

Abbreviations used in the Body of the Report:
2-DG - 2-deoxy-glucose
CI - combination index
ED - effective dose
FACs - Fluoresence-activated cell sorting
mCRPC - metastatic castrate-resistant prostate cancer
MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

Methods

Cell Culture: Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. LNCaP cells were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 1X antibiotic/antimycotic (Sigma), and 10% fetal bovine serum (FBS) (GIBCO-Invitrogen). PC-3 cells were grown in Nutrient Mixture F-12 Ham (F12K) (Sigma) supplemented with 1.1 g/L sodium bicarbonate, 1X antibiotic/antimycotic (Sigma), and 10% FBS (GIBCO-Invitrogen).

Drug incubations: Cells were plated 24 - 48 hrs before drug addition and were then maintained in the drug for 24 - 72 hrs. Docetaxel and metformin were from Tocris, paclitaxel from Molecular Probes, and nocodazole and 2-DG were from Sigma. Docetaxel, pactlitaxel and nocodazole stock solutions were prepared in DMSO; metformin and 2-DG stocks were prepared in dH_2O. For all experiments 0.1% DMSO was used as a vehicle control. The drug concentration ranges tested were based on preliminary experiments to explore concentration ranges where individual drugs inhibited cell proliferation and these preliminary experiments were used to estimate a median effective dose (ED50) for each drug in each cell line. Drug combination experiments were performed as described below using 4,2,1,0.5, and 0.25 times the median effective dose (ED50) of each drug in a constant ratio checkerboard design.

Cell proliferation assays: Cells were plated in 96-well plates at a density of 5×10^3 cells per well for either cell line. After 24 - 48 hrs, cells were treated with serial dilutions of individual drugs, or drug combinations, as noted. Control wells received an equivalent volume of assay medium containing 0.1% DMSO. All conditions were analyzed in triplicate within a single experiment, and all experiments were repeated 2 or more times, as noted. After 48 - 72 hr incubation, cell viability was quantified using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega) according to manufacturer's instructions. Briefly, 20 mL MTS solution was added to 100 mL medium per well, and plates incubated at 37°C for 1-2 hrs. Absorbance at 490 nm was measured using a Techan Infinite M200Pro plate reader driven by I-Control 1.7 software at 10 reads per well. Survival was calculated as a percent compared to control treated cells. To pool data from separate experiments, the absorbance value for DMSO treated cells was set to 2.0 and all values for each drug concentration scaled accordingly. This normalization procedure changed the absorbance value by relatively little since the DMSO treated cells typically had absorbance readings close to 2.0 in each individual experiment.

As a more direct measure of cell death, trypan blue exclusion assays were performed as described previously (11).

Statistical analysis of cell proliferation data were performed using unpaired t-tests with GraphPad Software (www.graphpad.com/quickcalcs/ttest1.cfm).

Drug synergies: The combination index (CI) was calculated for treatment pairs using CalcuSyn software (Biosoft, Ferguson,MO). The CI provides a quantitative measure of the degree of interaction between multiple agents (12). A CI of <1 denotes synergy, a CI of 1 denotes an additive effect, and a CI of >1 denotes antagonism. CI values were determined for each individual experiment and are given in the tables as the average of at least 3 experiments per treatment combination.

Indirect immunofluorescence and confocal microscopy: Cells were fixed and imaged as described previously (13). Microtubules were labeled with a mouse monoclonal α-tubulin (B512; Sigma-Aldrich) and a goat anti-mouse Alexa Fluor 488 (Invitrogen). Propidium iodide (0.15 μM) was used to label DNA. Cells were imaged by confocal microscopy as described previously (13) and images acquired using a 40X/1.3NA objective. Image stacks were converted to maximum intensity projections, exported as TIFF files and assembled using Photoshop.

Results and Discussion:

MT stabilizing drugs, docetaxel and paclitaxel, act synergistically with the metabolic inhibitor, metformin, to inhibit proliferation of LNCaP and PC-3 prostate cancer cell lines.

To address whether prostate cancer cell lines are more sensitive to microtubule-targeting drugs when combined with metabolic inhibition, we first assayed docetaxel, the primary treatment option for advanced, metastatic prostate cancer (1) in combination with metformin, a metabolic inhibitor (5,7). As shown in Figure 1 (A,B) for cells treated for 48 hr with either drug alone or in combination, LNCaP cells were more sensitive to the combination of docetaxel and metformin than to either drug alone (see Table 1 for drug concentrations and statistical analyses). PC-3 were also more sensitive to the drug combination at low concentrations, but at most of the concentrations tested, cell proliferation was inhibited primarily by metformin (see Table 1 for drug concentrations and statistical analyses). A longer incubation of 72 hr showed the same general trends (not shown), but we observed greater experiment-to-experiment variation at this time point, and therefore 48 hr incubations were used for most analyses. For each cell line, cells were more sensitive to increasing metformin concentrations than to increasing docetaxel concentrations for the ranges tested (Figure 1 A,B; Table 1). Additional experiments testing higher concentrations of docetaxel (up to 1.6 μM) did not cause significantly greater cell death than that shown in Figure 1.

The commonly used MTS assay for cell viability measures the activity of intracellular dehydrogenases as a proxy for cell health and cell number. Trypan blue uptake was then used as a more direct measure of cell death. As shown in Figure 1 C,D, docetaxel (50 nM; approximately one-half the ED50 for cell death) or metformin (1.5 mM; approximately one-half the ED50 for cell death) increased the percent of trypan blue positive LNCaP and PC-3 cells over that seen in DMSO treated controls. As expected, combining the two drugs increased the percentage of dead cells in each cell line.

To test quantitatively whether docetaxel and metformin act synergistically in LNCaP or PC-3 cells, CI values were calculated for cells treated with these drugs. As shown in Tables 2 and 3, the combination of docetaxel and metformin increased cell death synergistically at the ED50.

To confirm that docetaxel is acting by targeting microtubules, we asked whether paclitaxel, another microtubule-stabilizing drug, also acts synergistically with metformin to increase cell death. For each cell line, the drug combination was more effective at decreasing cell proliferation than either drug individually over a range of concentrations (Figure 2 and Table 4; measurements from 72 hr drug incubations). From these data the calculated CI value at the ED50 indicated synergy between paclitaxel and metformin in each cell (Tables 2,3).

Although we were able to measure synergy between microtubule stabilizing drugs and metformin, we were surprised by how relatively insensitive each cell line was to the microtubule-targeting drugs. Therefore, we next assayed cell cycle distributions to ask whether LNCaP and PC-3 cells were responding to docetaxel by blocking in mitosis. DNA content per cell was measured by flow cytometry. LNCaP cells incubated in 50 nM docetaxel (approximately one-half the ED50 for cell death) for 24 hrs showed a significant increase in the number of cells with 4N DNA content, but the cell cycle block was more pronounced in PC-3 cells, where nearly all cells had a 4N DNA content (Figure 3 A,B). For each cell line, incubation for 48 hours in docetaxel reduced the number of cells with 2N DNA content, and also produced a large increase in the number cells with <2N DNA content, consistent with increased numbers of dying cells (Figure 3 A,B). The percent of cells with <2N content rose from ~1% in DMSO-treated cells to 24±8% (LNCaP cells) and 11±1% (PC-3 cells).

Cells were also examined by fixation and staining for tubulin and DNA (Figure 4). In each cell line, docetaxel treatment resulted in formation of multipolar spindles and bundles of microtubules, consistent with the greater MT stabilization. Spindles assembled in DMSO-treated cells typically had a bipolar structure (85% of LNCaP spindles and 80% of PC-3 spindles). In contrast, 85% (LNCaP) and 91% (PC-3) of cells treated with docetaxel formed multipolar spindles. Therefore, both lines were responding to docetaxel with the expected mitotic block. Metformin treatment (1.5 mM) increased the percentage of LNCaP cells in G1 (2N DNA content; Figure 3); consistent with a previous report where LNCaP cells were treated with a higher concentration (5mM) of metformin (6). In PC-3 cells, 1.5 mM metformin did not alter the cell cycle distribution compared to DMSO-treated cells. For both cell lines, mitotic spindles assembled in metformin were of normal bipolar structure (Figure 4; 100% of LNCaP and PC-3 spindles).

Metformin also acts synergistically with a MT depolymerizing drug, nocodazole, to inhibit cell proliferation

To determine whether the observed synergy between MT stabilizing drugs and metformin resulted from microtubule stability (present throughout the cell cycle) or a cell cycle block during mitosis, we treated the two cell lines with nocodazole, which acts oppositely from docetaxel or paclitaxel and depolymerizes microtubules, but also blocks cells in mitosis. Each

cell line responded to 500 nM nocodazole with a block in G2/M, as measured by flow cytometry (Figure 5 A,B). Treatment of each cell line with a range of nocodazole or metformin concentrations inhibited cell proliferation (Figure 5 C,D; Table 5) with a profile similar to that observed with docetaxel or paclitaxel. As observed with the microtubule stabilizing drugs, nocodazole was only moderately effective at killing cells in these lines, at least after 48 hr incubation. Metformin treatment resulted in greater inhibition of cell proliferation before reaching a plateau. The CI value calculated from these data indicate synergy between nocodazole and metformin at the ED50 in LNCaP cells (Table 2). For PC-3 cells, metformin was as effective as metforming plus nocodazole at most concentrations tested (Table 5) and a CI value was not calculated. Qualitatively, cell proliferation was more sensitive to microtubule stabilizers (docetaxel or paclitaxel) than to nocodazole, although the mechanism responsible for this difference is not known. It is possible that increased microtubule stability relays intracellular signals in addition to those generated by activation of the spindle assembly checkpoint.

Docetaxel synergizes with 2-DG to inhibit cell proliferation

To further test the hypothesis that microtubule-targeting drugs act in synergy with metabolic inhibition, we tested a second metabolic inhibitor, 2-DG, a competitive inhibitor of hexokinase, which catalyzes the first step of glycolysis. As shown in Figure 6, incubation in 2-DG reduced cell proliferation over a range of concentrations from 5 - 80 mM in both LNCaP and PC-3 lines. In combination with docetaxel, 2-DG-treated cells showed reduced cell proliferation, which was observed most prominently at the lower drug levels tested (Figure 6; Table 6). The CI values at the ED50 for docetaxel and 2-DG indicated synergistic activity in both LNCaP and PC-3 cell lines (Tables 2,3). By flow cytometry we did not observe a cell cycle block in 2-DG (20 mM) treated cells (Figure 6), but treatment with both 2-DG and docetaxel resulted in a large fraction of cells remaining in G1, even in PC-3 cells, where docetaxel shifts almost all cells to G2/M phases within the 24 hour drug incubation (see Figure 3B). The flow cytometry data indicate that the combination of 2-DG and docetaxel may function synergistically by effecting more than one cell cycle stage. Others have reported cell cycle blocks in cells treated with 2-DG alone, but these blocks were observed after longer incubations (4 days, 5 mM), and varied between a G1 block and G2/M block, depending on cell line (14).

KEY RESEARCH ACCOMPLISHMENTS

- Docetaxel and metformin act synergistically to control LNCaP or PC-3 cancer cell line proliferation and promote cell death.

- Paclitaxel and metformin act synergistically to control LNCaP or PC-3 cancer cell line proliferation and promote cell death.

- Nocodazole and metformin act synergistically to control LNCaP cancer cell line proliferation and promote cell death. PC-3 cells were inhibited to no greater extent by the drug combination than to metformin treatment alone.

- Docetaxel and 2-DG act synergistically to control LNCaP or PC-3 cancer cell line proliferation and promote cell death.

REPORTABLE OUTCOMES
Manuscripts, Abstracts, Presentations:

Manuscript submitted to BMC Cancer. July 2013. Bruce K. Carney and Lynne Cassimeris. Microtubule-targeted drugs act in synergy with metabolic inhibitors, metformin or 2-deoxy-glucose, to control growth of LNCaP and PC3 prostate cancer cell lines.

Prompted by the reviewers to conduct additional analyses we elected to withdraw the manuscript from consideration at this time.

Abstract presented at annual meeting of the American Society for Cell Biology. Bruce K. Carney and Lynne Cassimeris. 2012. Docetaxel and metformin act synergistically to inhibit growth of prostate cancer cell lines. Mol. Biol. Cell. 23: 869. Presented by B.K. Carney, December 2012, San Francisco, CA.

Patents and licenses: None.

Degrees obtained: None.

Development of cell lines, tissues or serum repositories: None.

Informatics: None.

Funding applied for based on this work: None.

Employment/Research applied for and/or received. Dr. Carney is now an adjunct faculty member at Moravian College, Bethlehem, PA and Northampton Community College, Tannersville, PA.

Conclusions:

The synergies observed between MT-targeted drugs and two metabolic inhibitors indicate that these drug combinations could provide more effective tumor cell killing than either drug alone. In particular, docetaxel, currently the best chemotherapeutic option for mCRPC, was more effective at cell killing when combined with either metformin or 2-DG in two commonly used prostate cancer lines. In the experiments reported here, the two drugs were added simultaneously and it is possible that greater synergistic effects when drugs are added sequentially, as described recently for docetaxel plus vandetanib to inhibit VEGFR-2 tyrosine kinase (15). A dual metabolic inhibition, combining treatment with both metformin and 2-DG, also resulted in greater cell death in the LNCaP prostate cancer cell line (6). Another treatment showing synergy in prostate cancer cells combined docetaxel with oncolytic reovirus infection (16).
The molecular mechanisms underlying drug synergies observed in prostate cancer cell lines, combining docetaxel with either metabolic inhibitors or reovirus infection (16), are not known.

We attempted to test whether the synergies we observed were due to a mitotic block (produced by all of the microtubule-targeting drugs used here) or by a microtubule-dependent effect, independent of the cell cycle. LNCaP and PC-3 cells were treated with the cyclin-dependent kinase 1 inhibitor RO-3306 (17), which should block cell cycle progression just prior to mitotic entry. LNCaP were not blocked in mitosis by this drug, and in both cell lines we observed very little cell death after 48 h incubation in RO-3306, making it impossible to test for synergy with metabolic inhibitors. A mechanism independent of the cell cycle has been proposed from experiments in breast cancer cell lines and suggests that the combined treatment of paclitaxel and 2-DG induces cell death by causing oxidative stress (18). It is possible that a similar mechanism is responsible for the synergies observed in prostate cancer cells. Finally, it is also possible that reduced cellular metabolism (metformin or 2-DG treated cells) may reduce the ATP needed for drug efflux from cells.

A clinical trial to test the combination of metformin and docetaxel in mCRPC is currently ongoing (19). While our cell-based studies suggest that combination therapy will be successful clinically, we note that metabolic inhibition may not always be effective in all cancers or in all patients, as discussed by Yamaguchi and Perkins (20).

While our results generally support the idea that the combination of microtubule-targeted drugs and metabolic inhibitors act synergistically to inhibit prostate cancer cell proliferation in a super-additive way, several concerns about our data made us unwilling to publish the results as they stand now. First, the ED50 for all microtubule-targeted drugs tested here is more than an order of magnitude greater than that reported in previously published work (22). We cannot find a reasonable explanation for the different sensitivities found here compared to published data from the same cell lines. We eliminated potential technical errors (e.g. dilutions); we found that the paclitaxel ED50 in Hela cells falls within the expected range, indicating that we are conducting proliferation assays correctly; we purchased LNCaP and PC-3 lines from the ATCC specifically for these studies and are confident that these lines have not been contaminated by other human cells. Second, statistical analyses (see Tables) indicates that some drug combination data are not significantly different from either drug alone, reducing our confidence in our overall conclusion of synergy. While we do not have the confidence in the data to publish at this time, we are optimistic that drug combination therapy will prove successful to increase the efficacy of docetaxel treatment for metastatic prostate cancer.

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http://clinicaltrials.gov/ct2/show/NCT01796028

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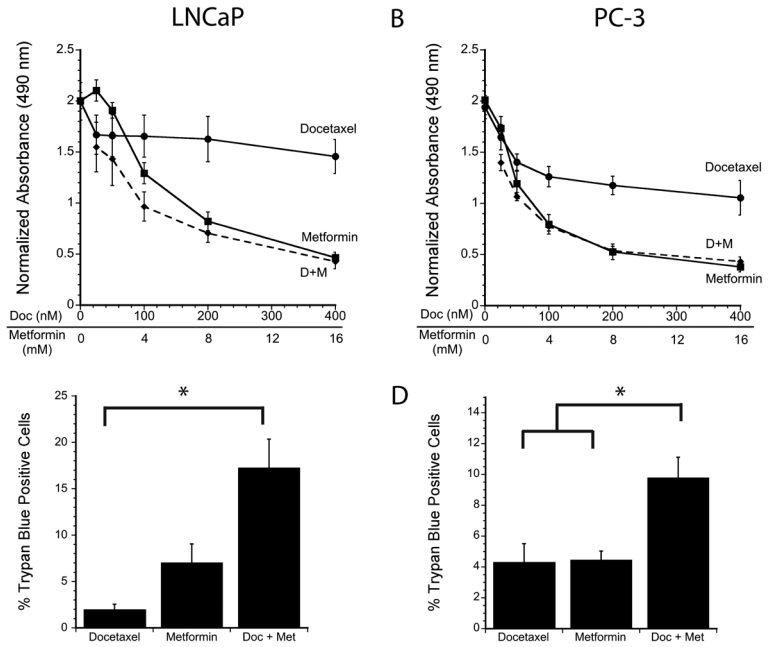
Supporting Data.

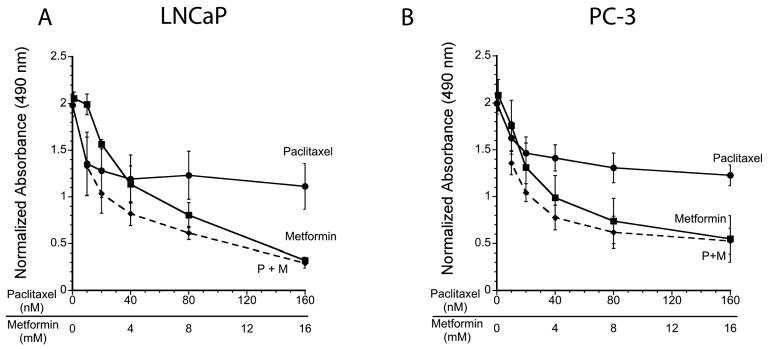
Figures and Legends:

Figure 1



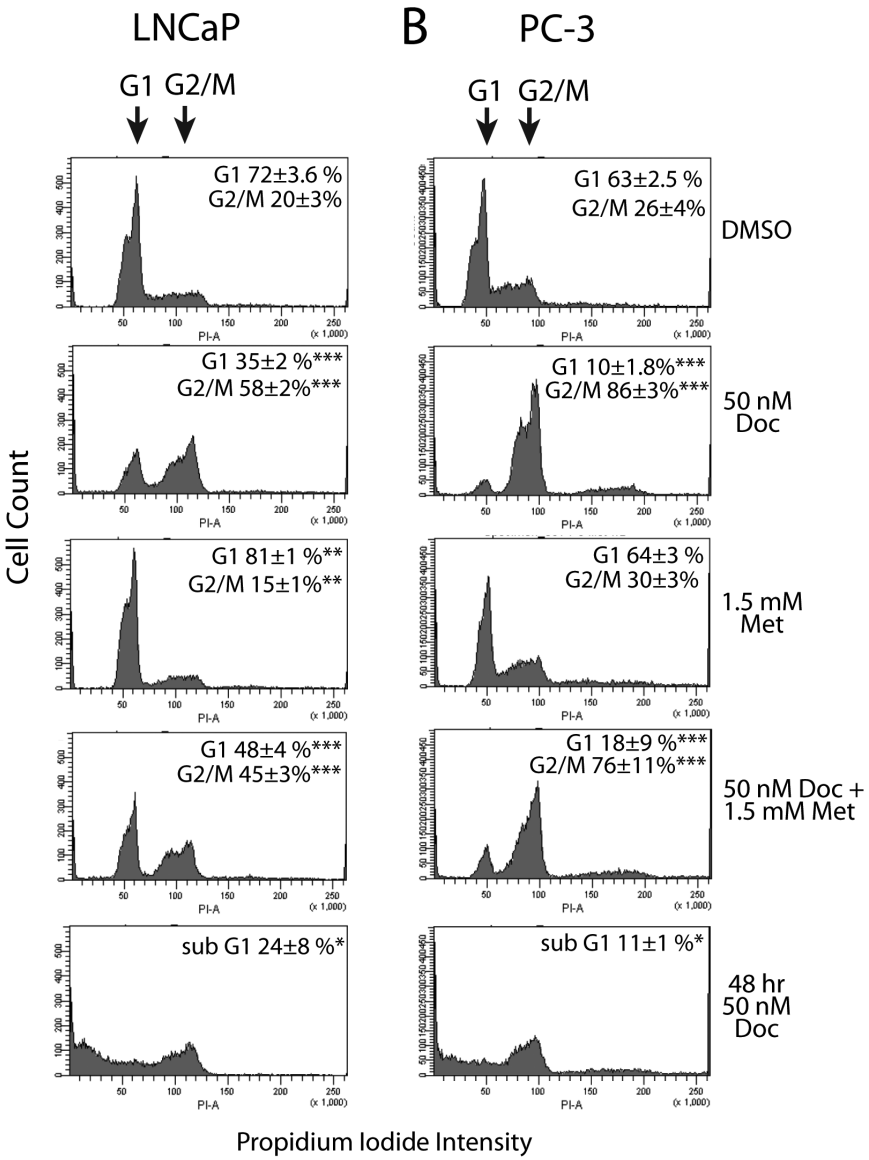
Legend: Metformin enhances the cell death caused by docetaxel treatment of LNCaP and PC-3 cell lines. (A, B) LNCaP (A) and PC-3 (B) cell lines were treated with docetaxel (0-400 nM), metformin (0-16 mM), or the two drugs in a constant ratio checkerboard design. For each experiment, each drug concentration or combination was tested in triplicate and cells were incubated for 48 hrs after addition of drugs. As a control, cells were incubated in 0.1% DMSO. Cell viability was measured by MTS assay and results were normalized to pool experiments. Plots show the combined data from 3 (LNCaP) or 4 (PC-3) independent experiments, additional experiments including different concentration ranges gave the same general patterns. (C, D) The percent cell death (Trypan blue positive cells) for LNCaP (C) and PC-3 (D) lines. Cells were incubated for 48 hrs in docetaxel (50 nM; approximately one-half the ED50 for cell death) and/or metformin (1.5 mM; approximately one-half the ED50 for cell death). The percent trypan blue positive cells measured in DMSO-treated samples was subtracted from each experimental condition. Doc, D = docetaxel; Met, M = metformin

Figure 2



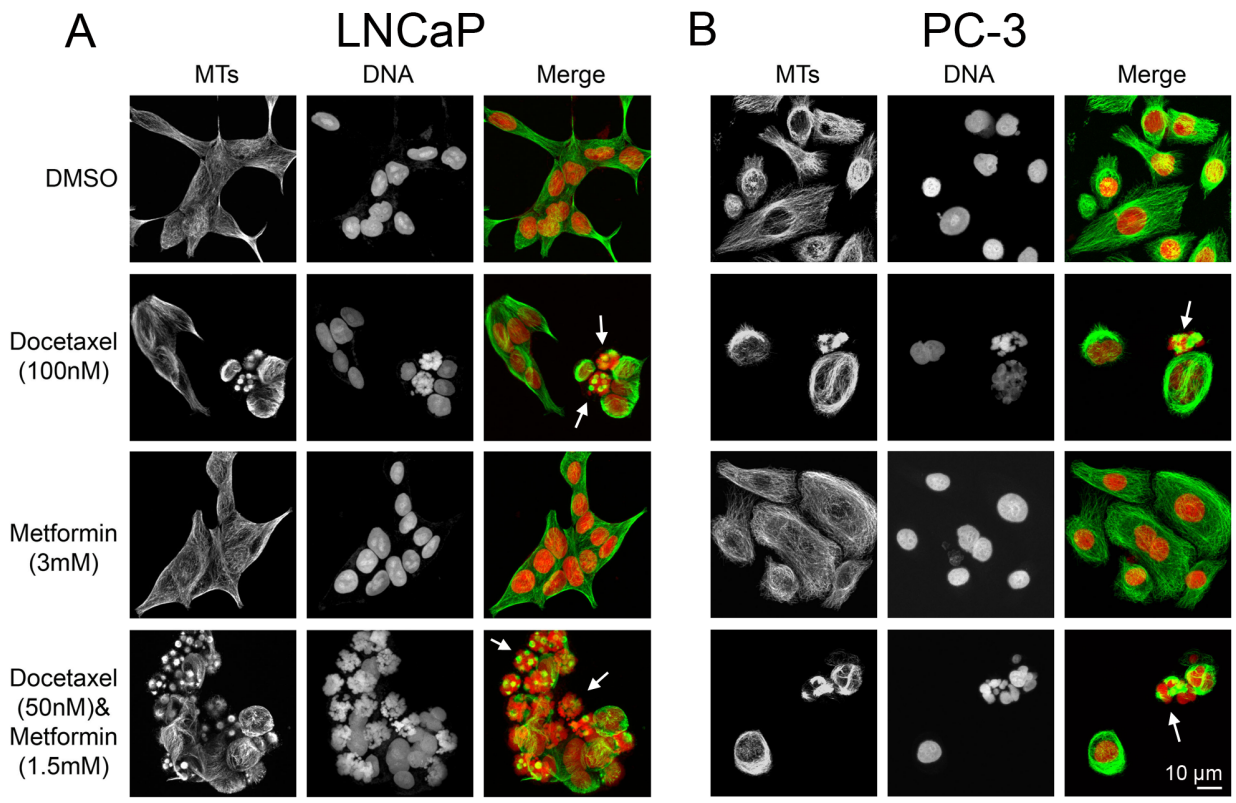
Legend: Metformin increases the cell death caused by paclitaxel in LNCaP and PC-3 cells. (A, B) LNCaP (A) and PC-3 (B) cell lines were treated with paclitaxel (0-160 nM), metformin (0-16 mM), or the two drugs in a constant ratio checkerboard design. Each drug concentration or combination was tested in triplicate in each experiment and cells were incubated for 72 hrs after addition of drugs. As a control, cells were incubated with 0.1% DMSO. Cell viability was measured by MTS assay and results from 3 independent experiments were normalized to pool experiments. P= paclitaxel; M = metformin

Figure 3



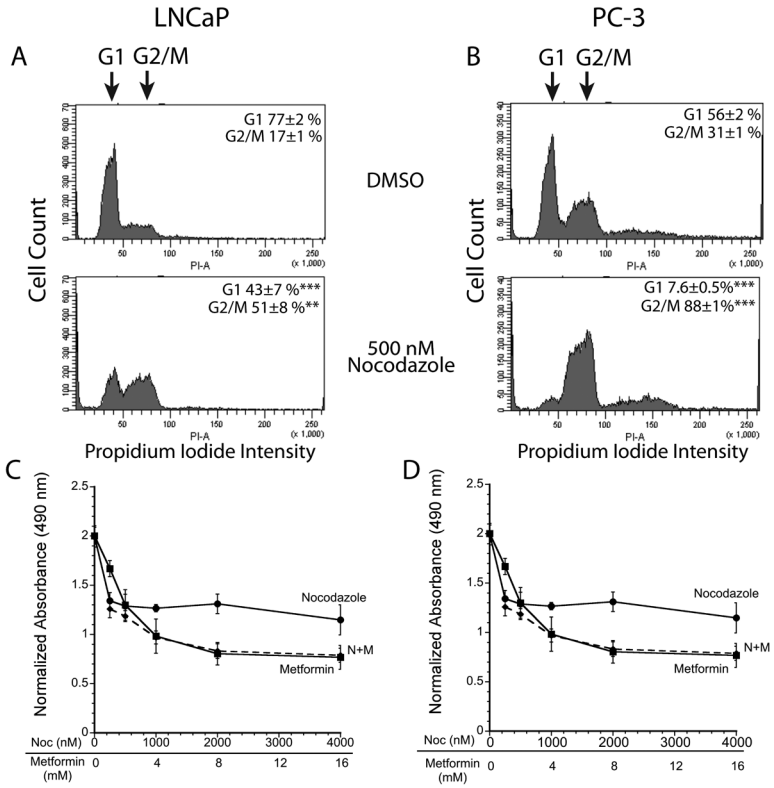
Legend: Cell cycle profiles of LNCaP and PC-3 cells treated with docetaxel and/or metformin. (A,B) DNA content of LNCaP (A) and PC-3 (B) cells measured by flow cytometry. Unless indicated otherwise, cells were incubated in drug containing medium for 24 hr. As a control, cells were incubated with 0.1% DMSO. Cells were fixed and DNA labeled with propidium iodide. Each experiment was repeated three times and plots shown are from representative experiments. Drug concentrations were approximately half of the estimated EC50 for each drug alone. Doc = docetaxel; Met = metformin.

Figure 4



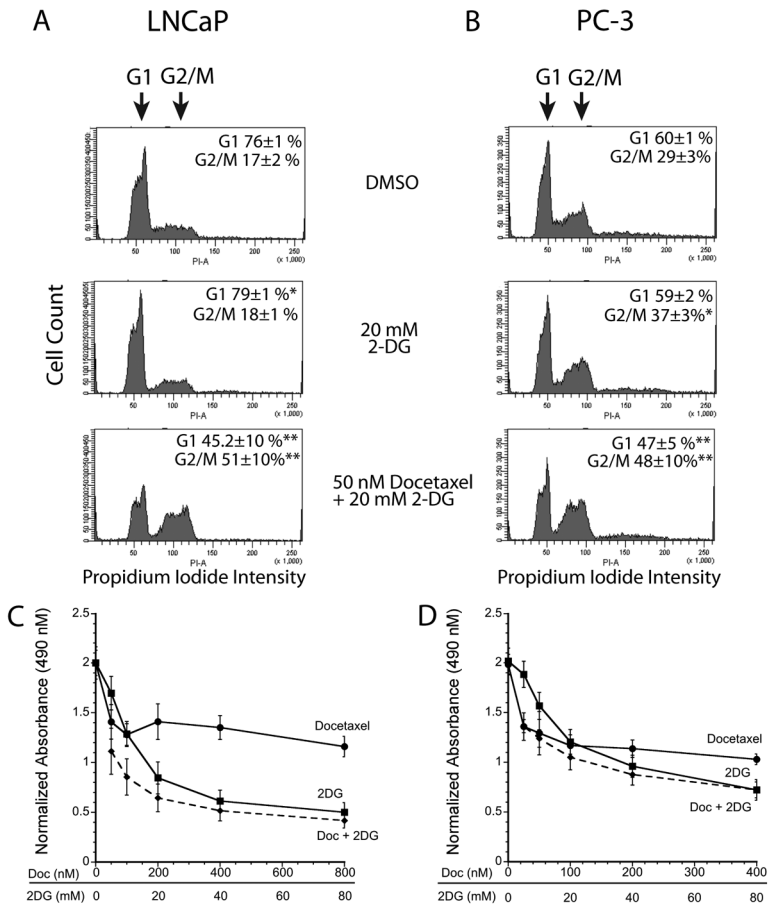
Legend: Docetaxel treatment resulted in multipolar spindle formation in LNCaP and PC-3 cells. Each cell line was incubated for 24 hr in DMSO, docetaxel or metformin and then fixed and stained with an antibody against α-tubulin (green in merged images) and propidium iodide (red in merged images). Representative images from each treatment are shown. Docetaxel, alone or in combination with metformin, resulted in a large increase in multipolar spindles (Arrows in merged images). Metformin-treated cells appeared to have MT organization indistinguishable from DMSO treated cells. Scale bar = 10 µm.

Figure 5



Legend: Metformin enhanced nocodazole-induced cell death in LNCaP and PC-3 cell lines. (A,B) DNA content, measured by flow cytometry, for LNCaP (A) and PC-3 (B) cells after 24 hr incubation in 500 nM nocodazole, a MT-depolymerizing drug. For each cell line, the peak at 4N DNA content (G2/M phases) was increased significantly by drug treatment, indicating that each line was responding with the expected mitotic block. (C,D) Cell number decreased significantly in cells treated with nocodazole (0-4000nM) and/or metformin (0-16mM). Each drug concentration was assayed in triplicate 48 hrs after drug addition. As a control, cells were incubated in 0.1% DMSO. Plots show data pooled from 3 independent experiments. At the lower concentrations tested, nocodazole and metformin caused slightly more cell death than either drug separately. Noc, N = nocodazole; M = metformin.

Figure 6:



Legend: 2-DG enhances the cell death caused by docetaxel in LNCaP and PC-3 cells. (A,B) DNA content of LNCaP (A) and PC-3 cells incubated for 24 hrs in the indicated concentrations of 2-DG and/or docetaxel. No significant change in cell cycle distribution was noted for either cell line treated with 2-DG, while the mitotic block (increased numbers of cells with 4N DNA content, labeled G2/M) was observed after incubation in the combination of docetaxel and 2-DG. Drug concentrations are approximately half the ED50 value. (C,D) Cell number measured by MTS assay for LNCaP (C) and PC-3 (D) cells after 48 hr incubation in the indicated drugs. As a control, cells were incubated in 0.1% DMSO. Plots show normalized data averaged for 4 experiments in LNCaP cells and 3 experiments in PC-3 cells. Additional experiments covering a broader concentration range showed the same patterns. Doc = docetaxel.

Supporting Data, Part 2

Tables

Table 1. Docetaxel and metformin drug inhibition of cell proliferation in LNCaP and PC-3 cells.

		LNCaP			PC-3	
Docetaxel (nM)	Metformin (mM)	MTS (OD 490 ± SD)	p value: individual drug vs. combination		MTS (OD 490 ± SD)	p value: individual drug vs. combination
0	0	2.01 ± 0.14			2.00 ± 0.13	
25	0	1.67 ± 0.19	NS		1.65 ± 0.12	<0.0001
50	0	1.66 ± .20	NS		1.40 ± 0.08	<0.0001
100	0	1.66 ± 0.20	<0.0001		1.26 ± 0.1	<0.0001
200	0	1.63 ± 0.22	<0.0001		1.17 ± 0.09	<0.0001
400	0	1.46 ± 0.17	<0.0001		1.05 ± 0.17	<0.0001
0	1	2.10 ± .10	<0.0001		1.73 ± 0.12	<0.0001
0	2	1.91 ± 0.08	<0.0001		1.19 ± 0.12	0.0022
0	4	1.29 ± 0.10	<0.0001		0.80 ± 0.1	NS
0	8	0.82 ± 0.09	0.015		0.53 ± 0.08	NS
0	16	0.46 ± 0.05	NS		0.38 ± 0.05	0.014
25	1	1.55 ± 0.24			1.40 ± 0.08	
50	2	1.43 ± 0.26			1.07 ± 0.04	
100	4	0.99 ± 0.12			0.77 ± 0.05	
200	8	0.70 ± 0.09			0.54 ± 0.04	
400	16	0.43 ± 0.07			0.43 ± 0.46	

Drug concentrations for docetaxel and metformin are given the in the two left columns. The p values listed compare MTS readings for an individual drug alone or in combination with the second drug. Data shown are for cells treated with indicated concentrations for 48 hr.

Table 2: Combination Index values for LNCaP cells treated with MT-targeted drugs and metabolic inhibitors

Drug combination	ED50	r^2	n
Docetaxel, Metformin	0.86 ± 0.1	0.98	5
Paclitaxel, Metformin	0.63 ± 0.2	0.95	3
Nocodazole, Metformin	0.79 ± 0.19	0.92	3
Docetaxel, 2-DG	0.87 ± 0.18	0.99	5

Combination index values ± SD for the drug combinations listed.
Number of independent experiments, n.

Table 3: Combination Index values for PC-3 cells treated with MT-targeted drugs and metabolic inhibitors.

Drug combination	ED50	r^2	n
Docetaxel, Metformin	0.67 ± 0.24	0.96	7
Paclitaxel, Metformin	0.72 ± 0.29	0.96	3
Nocodazole, Metformin	NS		
Docetaxel, 2-DG	0.37 ± 0.14	0.97	6

Combination index values ± SD for the drug combinations listed.
Number of independent experiments, n.

Table 4: Paclitaxel and metformin inhibition of cell proliferation in LNCaP and PC-3 cells

		LNCaP			PC-3	
Paclitaxel (nM)	Metformin (mM)	MTS (OD 490 ± SD)	p value: individual drug vs. combination		MTS (OD 490 ± SD)	p value: individual drug vs. combination
0	0	2.01 ± 0.1			2.03 ± 0.15	
10	0	1.36 ± 0.34	NS		1.63 ± 0.17	0.0017
20	0	1.28 ± 0.29	NS		1.46 ± 0.17	<0.0001
40	0	1.19 ± 0.26	0.0014		1.41 ± 0.14	<0.0001
80	0	1.23 ± 0.26	<0.0001		1.31 ± 0.16	<0.0001
160	0	1.11 ± 0.25	<0.0001		1.23 ± 0.11	<0.0001
0	1	1.99 ± 0.11	<0.0001		1.76 ± 0.27	0.0009
0	2	1.56 ± 0.05	<0.0001		1.31 ± 0.26	0.010
0	4	1.14 ± 0.20	0.0009		0.99 ± 0.24	0.033
0	8	0.80 ± 0.13	0.0016		0.73 + 0.24	NS
0	16	0.32 ± 0.04	NS		0.55 ± 0.25	NS
10	1	1.32 ± 0.31			1.36 ± 0.12	
20	2	1.04 ± 0.21			1.04 ± 0.09	
40	4	0.82 ± 0.13			0.78 ± 0.13	
80	8	0.61 ± 0.07			0.62 ± 0.17	
160	16	0.29 ± 0.06			0.52 ± 0.14	

Drug concentrations for paclitaxel and metformin are given the in the two left columns. The p values listed compare MTS readings for an individual drug alone or in combination with the second drug. Data shown are for cells treated with indicated concentrations for 72 hr.

Table 5: Nocodazole and metformin inhibition of cell proliferation in LNCaP and PC-3 cells.

		LNCaP			PC-3	
Nocodazole (nM)	Metformin (mM)	MTS (OD 490 ± SD)	p value: individual drug vs. combination		MTS (OD 490 ± SD)	p value: individual drug vs. combination
0	0	1.98 ± 0.18			2.00 ± 0.09	
250	0	1.05 ± 0.08	NS		1.34 ± 0.08	NS
500	0	0.94 ± 0.14	<0.0001		1.29 ± 0.12	0.034
1000	0	0.94 ± 0.13	<0.0001		1.27 ± 0.04	<0.0001
2000	0	0.92 ± 0.14	<0.0001		1.31 ± 0.1	<0.0001
4000	0	0.95 ± 0.15	<0.0001		1.15 ± 0.15	<0.0001
0	1	1.92 ± 0.16	<0.0001		1.67 ± 0.08	<0.0001
0	2	1.46 ± 0.11	<0.0001		1.30 ± 0.15	NS
0	4	0.82 ± 0.09	<0.0001		0.98 ± 0.17	NS
0	8	0.61 ± 0.75	0.0008		0.80 ± 0.11	NS
0	16	0.47 ± 0.09	NS		0.76 ± 0.12	NS
250	1	0.96 ± 0.13			1.26 ± 0.09	
500	2	0.71 ± 0.07			1.19 ± 0.06	
1000	4	0.52 ± 0.08			0.97 ± 0.07	
2000	8	0.47 ± 0.07			0.83 ± 0.07	
4000	16	0.40 ± 0.05			0.79 ± 0.12	

Drug concentrations for nocodazole and metformin are given the in the two left columns. The p values listed compare MTS readings for an individual drug alone or in combination with the second drug. Data shown are for cells treated with indicated concentrations for 48 hr.

Table 6: Docetaxel and 2DG inhibit LNCaP and PC3 cell proliferation

		LNCaP			PC-3	
Docetaxel (nM)	2DG (mM)	MTS (OD 490 ± SD)	p value: individual drug vs. combination		MTS (OD 490 ± SD)	p value: individual drug vs. combination
0	0	2.00 ± 0.14				
50	0	1.41 ± 0.17	0.0018		1.36 ± 0.14	<0.0001
100	0	1.28 ± 0.11	<0.0001		1.29 ± 0.22	<0.0001
200	0	1.41 ± 0.18	<0.0001		1.17 ± 0.12	0.0178
400	0	1.35 ± 0.12	<0.0001		1.14 ± 0.09	NS
800	0	1.16 ± 0.1	<0.0001		1.03 ± 0.05	NS
0	5	1.70 ± 0.17	<0.0001		1.88 ± 0.13	<0.0001
0	10	1.29 ± 0.13	<0.0001		1.57 ± 0.14	<0.0001
0	20	0.84 ± 0.16	0.0035		1.21 ± 0.12	.0178
0	40	0.61 ± 0.11	0.0358		0.96 ± 0.11	NS
0	80	0.50 ± 0.10	0.0308		0.72 ± 0.10	NS
50	5	1.11 ± 0.23			1.36 ± 0.07	
100	10	0.85 ± 0.18			1.24 ± 0.09	
200	20	0.64 ± 0.14			1.05 ± 0.13	
400	40	0.52 ± 0.10			0.88 ± 0.11	
800	80	0.42 ± 0.75			0.72 ± 0.08	

Drug concentrations for nocodazole and metformin are given the in the two left columns. The p values listed compare MTS readings for an individual drug alone or in combination with the second drug. Data shown are for cells treated with indicated concentrations for 48 hr.